Decreased Protein-Stabilizing Effects of Cryoprotectants Due to Crystallization

Ken-ichi Izutsu,[1,2] Sumie Yoshioka,[1] and Tadao Terao[1]

Received October 7, 1992; accepted January 21, 1993

The stabilizing effects of various additives against inactivation of an enzyme (β-galactosidase from *Aspergillus oryzae*) during freeze-drying were studied, with a focus on their crystallinity. The crystalline morphology of mannitol and inositol in freeze-dried cakes depended on the solute concentrations before freezing and the freeze-drying method used. The additives in their amorphous state showed concentration-dependent stabilization of the enzyme, whereas additive crystallization during freeze-drying decreased their effects. Heat treatment before freeze-drying also caused crystallization and diminished the stabilizing effects. Noncovalent soluble aggregates were observed in the inactivated enzyme solution. These results show the importance of maintaining the amorphous state of additives used as stabilizing agents during freeze-drying.

KEY WORDS: freeze-drying; protein-stabilizing effects; cryoprotectants; crystallization; β-galactosidase; mannitol.

INTRODUCTION

The use of biotechnology has resulted in the production of proteins for pharmaceutical purposes. However, proteins are chemically and physically unstable, which causes problems during their purification, formulation and storage (1). Freeze-drying is often used for protein formulations to achieve long-term stability (2,3). Although the shelf lives may be improved by freeze-drying, some proteins are inactivated during this process. Additives, including sugars, amino acids, and surfactants, have been used to prevent inactivation during freeze-drying (4–7), and relevant mechanisms were discussed (4,8). Sugars and amino acids protect proteins in solution against heat denaturation thermodynamically by preferential exclusion (8,9), which may also contribute a protective effect during freeze-thawing (4). Ionic additives, such as amino acids, protect enzymes by the preferential exclusion mechanism and by changing the pH of frozen solutions. Among the many effective additives used for freeze-thawing (termed cryoprotectants), some are ineffective for freeze-drying. In addition to preferential exclusion in frozen solutions, molecular interactions between proteins and additives are important for stabilization during freeze-drying. Studies using Fourier-transform infrared spectroscopy (FT-IR) have indicated the presence of hydrogen-bonding between proteins and stabilizing carbohydrates in freeze-dried cakes (10).

Crystallization of some additives (e.g., mannitol) and of various salts has been observed in freeze-dried cakes (11). Without crystallization, solutes keep their amorphous state during freeze-drying. As additives that tend to be amorphous possess protective activity, the maintenance of amorphism has been discussed as an essential property for stabilization. For example, sugars and hydroxypropyl-β-cyclodextrin, which remain amorphous in freeze-dried cakes, protect recombinant human growth hormone (5), whereas some amino acids and mannitol, which crystallized during freeze-drying, were less protective. Crystallization is believed to remove the solutes from the protein phase and result in the loss of molecular interaction with proteins (3,12). Maintenance of the amorphous state is also important to stabilize proteins during storage (13–15).

In this study, the relationship between the crystallization of mannitol, inositol, and glucose and their stabilizing effects on β-galactosidase from *Aspergillus oryzae* was studied. Freeze-dried samples, which crystallized to varying extents, were prepared by altering the additive and phosphate buffer concentrations and freeze-drying method.

Glucose usually remains amorphous during the freeze-drying process, whereas mannitol and inositol tend to crystallize (11,16). The stabilizing effect of mannitol during freeze-drying of hemoglobin (17) and L-asparaginase from *Erwinia carotovola* (18) was insufficient compared to other cryoprotectants.

MATERIALS AND METHODS

Materials

Lyophilized β-galactosidase powder from *Aspergillus oryzae* was purchased from Toyobo. Co. (Osaka, Japan). The polypropylene tubes used for freeze-drying were from Iuchi Seieido Co. (Osaka). Seamless cellulose tubing was purchased from Sanko Pure Chemical Co. (Tokyo). Mannitol, inositol, glucose, and other chemicals used were purchased from Wako Pure Chemical Ind. Ltd. (Osaka).

Freeze-Drying of β-Galactosidase

β-Galactosidase was dissolved to about 10 mg/mL in distilled water or sodium phosphate buffer (10 to 200 m*M*, pH 7.4) and dialyzed overnight. The enzyme solution was filtered through a 0.45-μm Millipore filter, and its protein concentration was determined using the method of Lowry *et al.* (19) with bovine serum albumin as the protein standard. Appropriate amounts of the enzyme and additive solutions were transferred to polypropylene tubes (flat-bottomed; bottom area, 3.46 cm^2) and frozen by immersing the tube in liquid nitrogen for at least 1 min. The frozen samples were transferred to the shelf of a freeze-drier (Freezevac-1CFS, Tozai Tsusho Co., Tokyo), which had been precooled to −40°C, and dried by maintaining the shelf temperature at −40°C for 1 hr, −35°C for 12 hr, and 35°C for 4 hr (Method A in Fig. 1). The temperature was changed at 1°C/min. β-Galactosidase solutions were also prepared and freeze-dried using potassium phosphate buffer (10 to 200 m*M*, pH 7.4) to investigate the effects of buffer salts.

Heat Treatment (Annealing) Before Freeze-Drying

In the experiments in which the effects of annealing were investigated, frozen samples were heat-treated on the

[1] National Institute of Hygienic Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158, Japan.
[2] To whom correspondence should be addressed.

0724-8741/93/0800-1232$07.00/0

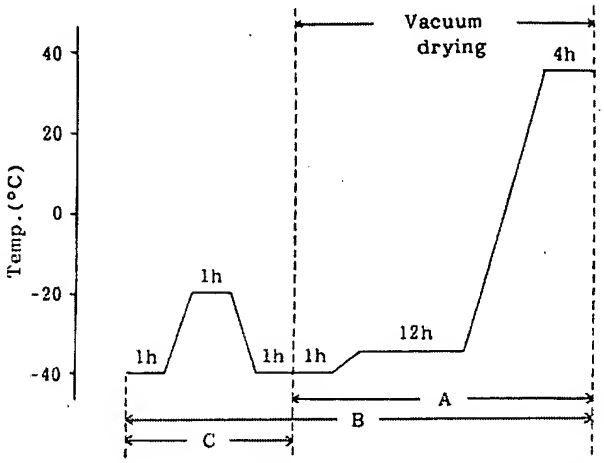

Fig. 1. Shelf temperature control during heat treatment and freeze-drying. Samples were freeze-dried or heat-treated by controlling the shelf temperature of the freeze-drier as described under Materials and Methods. Each method represents (A) freeze-drying, (B) freeze-drying after heat treatment, and (C) freeze-thawing after heat treatment.

shelf of the freeze-drier, by maintaining the shelf temperature at −40°C for 1 hr, −20°C for 1 hr, and −40°C for 1 hr, followed by freeze-drying as described above (Method B in Fig. 1). The shelf was heated and cooled at 1°C/min. Some samples were removed from the freeze-drier without being dried and assayed after melting, in order to study the effects of freeze-thawing after heat treatment (Method C in Fig. 1).

X-Ray Powder Diffraction

X-ray powder diffraction measurements were carried out on the freeze-dried cakes using a Rigaku RAD-2C system (Tokyo) with Ni-filtered Cu–Kα radiation (30 kV, 10 mA) at a scanning rate of 2°C/min.

Thermal Analysis of Crystallization

Differential scanning calorimetry (DSC) measurements were performed using a Shimadzu DSC-41M (Kyoto, Japan) to analyze the additive crystallization in frozen solutions. Aliquots (25 μL) of each sample solution were packed in aluminium cells, cooled to −60°C with liquid nitrogen, then heated at 2°C/min to 25°C, and the thermal changes were monitored using microcomputer software (Shimadzu).

Enzyme Assay

The freeze-dried enzyme samples were reconstituted with distilled water, and the enzyme activities were determined, as described previously (20). The residual activity was calculated as a percentage of that in the sample solution before freezing.

Residual Water in Freeze-Dried Cakes

Mannitol (100 m*M*) was dissolved in 50 m*M* sodium phosphate buffer solution containing the enzyme (2 μg/mL), and the solutions were freeze-dried after or without heat treatment. Each freeze-dried cakes was suspended in 2 mL methanol and the residual water content was determined using a Metrohm Karl Fisher coulometer (E-684, Herisaw, Switzerland).

High-Performance Size-Exclusion Chromatography (HPSEC) and Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The enzyme solutions (0.2 mg/mL) in sodium phosphate buffer (50 m*M*) containing 300 m*M* mannitol were freeze-dried, reconstituted with water to the same concentration. The reconstituted solutions were subjected to HPSEC and SDS-PAGE, as described elsewhere (20).

RESULTS AND DISCUSSION

Additive Crystallization in Freeze-Dried Cakes

Typical X-ray powder diffraction patterns of the freeze-dried products containing mannitol are shown in Fig. 2. As reported elsewhere (21), when aqueous solutions were freeze-dried the mannitol crystallized (Figs. 2A–C). The X-ray diffraction patterns show the different polymorphic forms in the freeze-dried cakes prepared from mannitol solutions of different concentrations. Although the β form (22) of D-mannitol predominated in the cakes freeze-dried from 100 and 200 m*M* mannitol, the α form, which has a characteristic peak at 9°, was present in the cake prepared from 500 m*M* mannitol. The crystallinity of the freeze-dried cakes containing sodium phosphate buffer differed depending upon the concentrations of mannitol and buffer salts (Figs. 2D–I). Mannitol did not crystallize when freeze-dried from relatively high buffer concentrations. It is not clear from these results whether the mannitol crystallized during the first (in frozen solution) and/or the second drying procedure.

Crystallization peaks of the sodium phosphate buffer salts were observed in the X-ray diffraction patterns of freeze-dried cakes prepared from buffer solutions with no other additives (data not shown). However, the sodium phosphate peaks were not detected in the cakes freeze-dried with the additives, due to the much larger crystalline peaks of the additives.

Figure 3 shows the DSC scans of frozen enzyme solutions, which contained 200 m*M* mannitol and various buffer concentrations. It has been reported that mannitol in water shows an exothermic peak at about −25°C due to crystallization of mannitol (16,21,23). We observed that the exothermic peak temperatures and structures in the presence of sodium phosphate buffer differed from that in aqueous solution. Although it is difficult to interpret these altered peaks, the thermal behavior changes may be related to the changes in crystallinity shown in Fig. 2.

Effect of Mannitol on the Residual Activity of the Freeze-Dried Enzyme

The effects of mannitol on the residual activities of β-galactosidase freeze-dried from solutions containing various concentrations of sodium phosphate buffer are shown in Fig. 4. The enzyme lost most of its activity during freeze-drying of mannitol-free buffer solutions. Mannitol protected the enzyme during freeze-drying, and its effects were dependent upon its concentration and that of the buffer salts. Mannitol

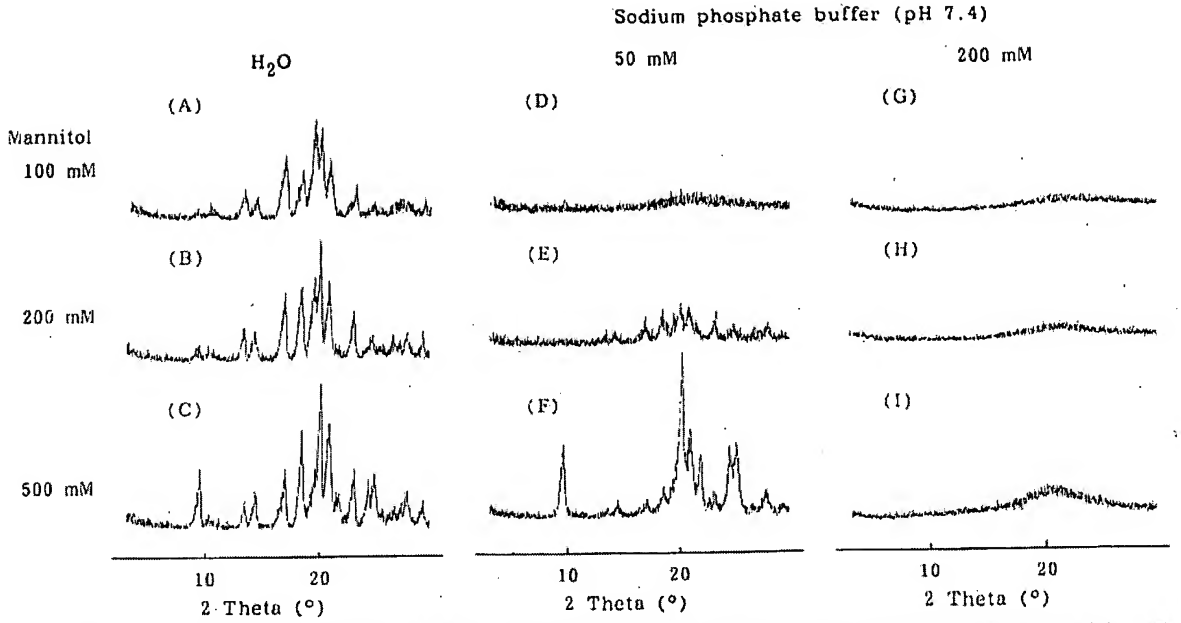

Fig. 2. Powder X-ray diffraction patterns of freeze-dried β-galactosidase. β-Galactosidase (2 μg/mL) solutions containing 100 m*M* (A, D, and G), 200 m*M* (B, E, and H), and 500 m*M* (C, F, and I) mannitol were freeze-dried. The freeze-dried cakes were obtained from simple aqueous solutions (A–C) and 50 m*M* (D–E) and 200 m*M* (G–I) sodium phosphate buffer (pH 7.4) solutions.

in 200 m*M* buffer stabilized the enzyme in a concentration-dependent manner. Most of the enzyme activity was preserved by the addition of 200–500 m*M* mannitol to the 200 m*M* buffer, and the mannitol in all the cakes freeze-dried from the buffer solution was amorphous. Mannitol in 10 and 50 m*M* buffer also exerted a stabilizing effect, which was maximal at 50–100 m*M* mannitol. Mannitol crystals were observed in the cakes freeze-dried from solutions containing mannitol in excess of its most effective concentration. Mannitol showed no stabilizing effect when a simple aqueous enzyme solution was freeze-dried, and it crystallized in all the cakes. These results show that mannitol in its amorphous state has a stabilizing effect on protein during freeze-drying, but it is less effective in the crystalline state. Similar results were obtained when a 20 μg/mL enzyme solution was freeze-dried (data not shown).

The sodium phosphate buffer acidifies in frozen solutions, and its pH decreases to 2–3, which causes enzyme

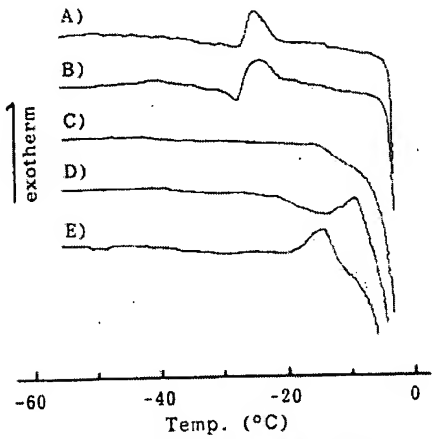

Fig. 3. The DSC scans of frozen solution. β-Galactosidase (2 μg/mL) solutions containing mannitol (200 m*M*) in the presence of sodium phosphate buffer (pH 7.4) were scanned at a scanning rate of 2°C/min. Buffer concentrations: 0 (A), 10 (B), 50 (C), 100 (D), and 200 (E) m*M*.

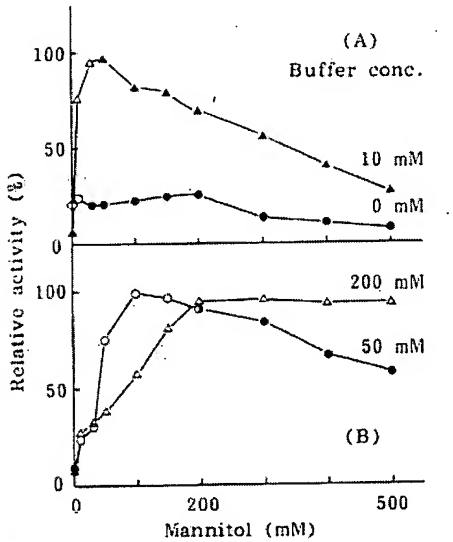

Fig. 4. Effect of mannitol on the residual activity of freeze-dried β-galactosidase. Aqueous solutions of the enzyme (2 μg/mL) containing no (◇, ○, and ● in A), 10 m*M* (△ and ▲ in A), 50 m*M* (○ and ● in B), and 200 m*M* (△ and ▲ in B) sodium phosphate buffer solution were freeze-dried. The open and filled symbols represent amorphous and crystallized cakes, respectively, analyzed by X-ray diffraction (◑: crystallization was observed in some samples). The open diamond denotes a collapsed cake which could not be analyzed by X-ray diffraction. The values are means of three separate experiments.

inactivation (24). We also studied the effects of mannitol in potassium phosphate buffers (10, 50, and 200 m*M*, pH 7.4), which are known to undergo smaller pH changes during freezing than sodium phosphate buffers (25). Solutions containing potassium phosphate buffer tend to collapse, especially when the concentration of mannitol was low. The enzyme freeze-dried without mannitol from the potassium containing buffer had a slightly higher residual activity than that from the sodium phosphate buffer. However, the relationships between the stabilizing effect and the crystallinity of mannitol were similar in the samples freeze-dried from both buffers (data not shown).

Effect of Heat Treatment (Annealing) on the Freeze-Drying and Freeze-Thawing of the Enzyme

When formulating freeze-dried pharmaceuticals, crystalline drugs are often desirable for shelf-life stability. Heat treatment of frozen solutions before drying is known to crystallize antibiotics (16). With this method, the frozen solutions are warmed to just above the crystallization exotherm temperature and then cooled before freeze-drying. The freeze-dried product of cephalothin sodium pretreated in this manner was crystalline, whereas it was amorphous when untreated. This technique is called aging, or annealing.

We used the heat-treatment method (Method B in Fig. 1) to study the effect of mannitol crystallization. The X-ray diffraction patterns obtained (Fig. 5) show that the ratio of crystalline to amorphous mannitol in the freeze-dried cakes was increased by heat treatment. The enzyme activities of the freeze-dried samples remaining after heat treatment are shown in Fig. 6A. Mannitol was crystallized in most of the cakes when the enzyme in 50 m*M* sodium phosphate buffer was heat-treated and freeze-dried, and the activities of these samples were lower than those not subjected to heat treatment. Heat treatment prior to freeze-drying caused mannitol crystallization and enzyme inactivation in the freeze-dried cakes. It is interesting that with 10 m*M* mannitol, which remained amorphous, the enzyme activity was higher than that in the other crystallized samples.

Although crystallized mannitol exerted a lesser stabilizing effect than the amorphous form, the activities remaining varied among the samples studied. Therefore the degree of crystallinity, type of polymorphic forms of mannitol, and stage at which they crystallize during the process also appear to be important factors for protein stabilization.

To test whether enzyme inactivation occurs during heat treatment or desiccation, we measured the enzyme activity of heat-treated frozen solutions which were not subjected to subsequent freeze-drying (Method C in Fig. 1). The effects of mannitol in the heat-treated freeze-thawed solutions are shown in Fig. 6B. The enzyme in both aqueous and buffer solutions lost some of its activity during the freeze-thawing process, but these losses were smaller than those of the enzyme solutions that were freeze-dried after heat treatment, which suggests that both the crystallization and the desiccation processes contribute to the inactivation.

Effects of Inositol and Glucose

The stabilizing effects of inositol and glucose were also studied. Glucose, like many sugars, is amorphous when freeze-dried, whereas inositol often crystallizes during freeze-drying. The effects of inositol on freeze-drying and

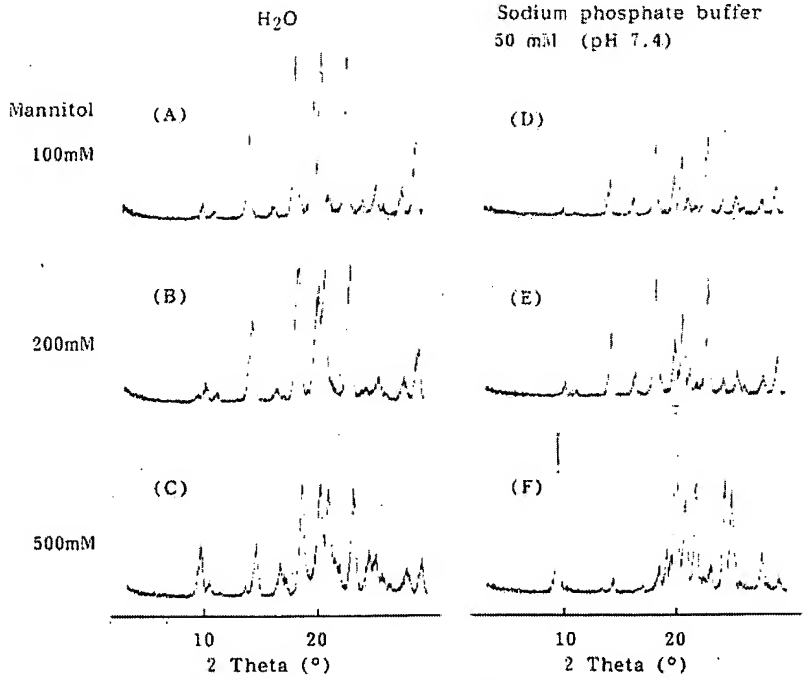

Fig. 5. Powder X-ray diffraction patterns of heat-treated and freeze-dried β-galactosidase. β-Galactosidase (2 μg/mL) solutions containing 100 m*M* (A, D), 200 m*M* (B, E), and 500 m*M* (C, F) mannitol were heat-treated and freeze-dried. The cakes were prepared from simple aqueous (A–C) and 50 m*M* sodium phosphate buffer (D–F) solutions.

freeze-thawing are shown in Fig. 7. When the enzyme was freeze-dried from sodium phosphate buffer solution (50 m*M*) containing inositol, a concentration-dependent stabilizing effect was observed and all the cakes were amorphous (Fig. 7A). When heat-treated before drying, inositol was crystallized in some samples, and the residual enzyme activities decreased, especially in the crystallized cakes (Fig. 7B). Low concentrations (30–100 m*M*) of inositol, which remained amorphous even after heat treatment, had a greater stabilizing effect than it did in the other samples. The enzyme activity also decreased in the heat-treated and freeze-thawed samples (Fig. 7C), but the enzyme lost most of the activity in the drying process. The inositol in the cakes freeze-dried from simple aqueous solutions crystallized (Fig. 7A). These cakes retained about 50% of the original enzyme activity after freeze-drying, which was reduced further by heat treatment.

Glucose protected the enzyme activity effectively during freeze-drying after and without heat treatment (data not shown), and the glucose in all the freeze-dried cakes was amorphous. When the minimum concentration of glucose required to preserve the enzyme activity was exceeded, the stabilizing effect reached a plateau and no activity decrease with increased glucose concentration was observed. The apparent enzyme activity increased by up to 20% when inositol or glucose was added to the assay solution, which may explain why the residual activities of some samples exceeded 100%.

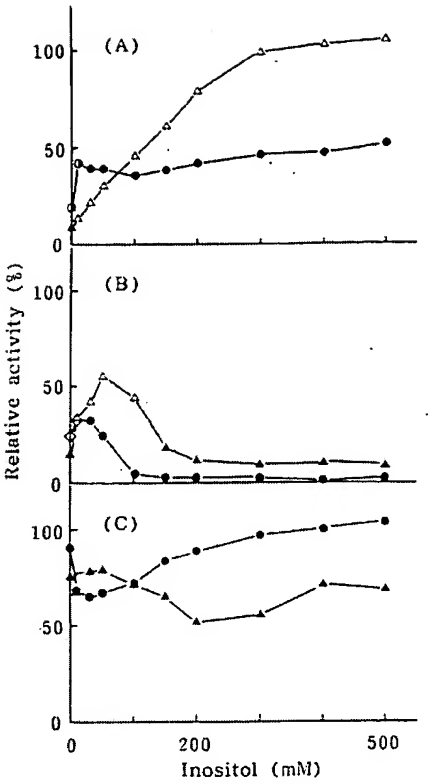

Fig. 7. Effect of inositol on the enzyme activity remaining after freeze-drying (A), freeze-drying after heat treatment (B), and freeze-thawing with heat treatment (C). The enzyme in simple aqueous (○, ●, ◇) and 50 m*M* sodium phosphate buffer (△, ▲) solutions were freeze-dried after (B) or without (A) heat treatment. The open and filled symbols represent amorphous and crystallized cakes, respectively, analyzed by X-ray diffraction (◑: crystallization was observed in some samples). The open diamond denotes a collapsed cake which could not be analyzed by X-ray diffraction. (C) The effects of freeze-thawing after heat treatment on the residual enzyme (2 μg/mL) activity in aqueous (●) and 50 m*M* sodium phosphate (▲) buffer solutions. The values are means of three separate experiments.

Residual Water in the Freeze-Dried Cakes

It has been suggested that enzyme inactivation during freeze-drying is due to deprivation of essential water (over-

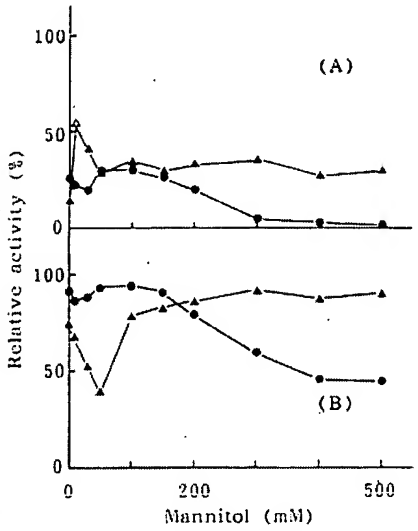

Fig. 6. Effect of mannitol on the residual activity of β-galactosidase freeze-dried after heat treatment (A) and freeze-thawed after heat treatment (B). (A) The residual enzyme activity of β-galactosidase (2 μg/mL) in 0 m*M* (●) and 50 m*M* (△, ▲) sodium phosphate buffer containing various concentrations of mannitol freeze-dried after heat treatment. The open and filled symbols represent amorphous and crystallized cakes, respectively, analyzed by X-ray diffraction. (B) The effect of freeze-thawing after heat treatment on the residual enzyme (2 μg/mL) activity in 0 m*M* (●) and 50 m*M* (▲) buffer. The values are means of three separate experiments.

drying). The relationship between the stabilizing effect of mannitol and the residual water of freeze-dried cakes was investigated by comparing the residual water in crystalline cakes freeze-dried after heat treatment with that in amorphous cakes freeze-dried from the same solution (100 m*M* mannitol in 50 m*M* sodium phosphate buffer) without heat treatment. The residual water (mean ± SD) in the crystallized cakes prepared after heat treatment was 1.73 ± 0.30%, and that in the amorphous cakes prepared without heat treatment was 1.06 ± 0.27%, which indicates that the amorphous cakes with higher enzyme activity contained less residual water. Therefore, the water content of the cakes does not appear to be related to the protein-stabilizing effect of mannitol.

HPSEC and SDS-PAGE of the Freeze-Dried Enzyme

The physical changes of the enzyme that occurred during freeze-drying were studied by carrying out HPSEC and SDS-PAGE on reconstituted solutions of freeze-dried enzyme. The results of a previous study showed that the en-

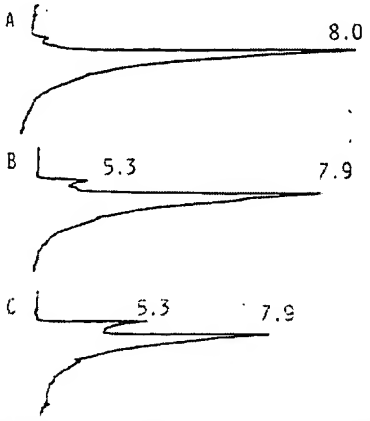

Fig. 8. High-performance size-exclusion chromatograms of β-galactosidase solution. Enzyme solutions (0.2 mg/mL) in sodium phosphate buffer (50 m*M*) containing 300 m*M* were subjected to HPSEC analysis. Solution before freeze-drying (A); reconstituted solutions of freeze-dried cake after (C) and without (B) heat treatment. The numbers beside peaks represent the retention times.

zyme formed noncovalent soluble aggregates when freeze-dried without additives (20). Mannitol crystallization and enzyme inactivation were observed when solutions (0.2 mg/mL) containing 50 m*M* phosphate buffer and 300 m*M* mannitol were freeze-dried. Figure 8 shows the typical HPSEC chromatograms of the reconstituted enzyme solutions. A peak due to soluble aggregates appeared before the original peak. The degree of aggregation was increased with the heat treatment. Aggregation by covalent bonding between the enzyme molecules and fragmentation were not observed with SDS-PAGE (data not shown). These results show that the inactivation of β-galactosidase during freeze-drying of the mannitol-containing solution was due to denaturation followed by formation of noncovalent soluble aggregates.

CONCLUSION

Amorphous mannitol, inositol, and glucose stabilized β-galactosidase against inactivation during freeze-drying. Mannitol and inositol lost their stabilizing effects when they crystallized. The enzyme inactivation appear to be due to denaturation that led to noncovalent aggregation.

REFERENCES

1. M. C. Manning, K. Patel, and R. T. Bochardt. Stability of protein pharmaceuticals. *Pharm. Res.* 6:903–918 (1989).
2. M. J. Pikal. Freeze-drying of proteins. I. Process design. *Biopharm.* 3:18–25 (1990).
3. M. J. Pikal. Freeze-drying of proteins. II. Formulation selection. *Biopharm.* 3:26–29 (1990).
4. T. Arakawa, Y. Kita, and J. F. Carpenter. Protein-solvent interactions in pharmaceutical formulations. *Pharm. Res.* 8:285–291 (1991).
5. M. J. Pikal, K. M. Dellerman, M. L. Roy, and R. M. Riggin. The effects of formulation variables on the stability of freeze-dried human growth hormone. *Pharm. Res.* 8:427–436 (1991).
6. M. E. Ressing, W. Jiskoot, H. Talsma, C. W. van Ingen, E. C. Beuvery, and D. J. A. Crommelin. The influence of sucrose, dextran, and hydroxypropyl-β-cyclodextrin as lyoprotectants for a freeze-dried mouse IgG_{2a} monoclonal antibody (MN12). *Pharm. Res.* 9:266–270 (1992).
7. T. Tamiya, N. Okahashi, R. Sakuma, T. Aoyama, T. Akahane, and J. J. Matsumoto. Freeze denaturation of enzymes and its prevention with additives. *Cryobiology* 22:446–456 (1985).
8. J. F. Carpenter and J. H. Crowe. The mechanism of cryoprotection of proteins by solutes. *Cryobiology* 25:244–255 (1988).
9. T. Arakawa and S. N. Timasheff. Stabilization of protein structure by sugars. *Biochemistry* 21:6536–6544 (1982).
10. J. F. Carpenter and J. H. Crowe. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochemistry* 28:3916–3922 (1989).
11. F. Franks. Improved freeze-drying: An analysis of the basic scientific principles. *Process Biochem.* 24:S3–S6 (1989).
12. A. P. MacKenzie. Freeze-drying of aqueous solutions containing peptides and proteins. In D. Marshak and D. Liu (eds.). *Therapeutic Peptides and Proteins: Formulations, Delivery, and Targeting. Current Communications in Molecular Biology*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989, pp. 17–21.
13. M. S. Hora, R. K. Rana, and F. W. Smith. Lyophilized formulations of recombinant tumor necrosis factor. *Pharm. Res.* 9:33–36 (1992).
14. M. J. Pikal, K. Dellerman, and M. L. Roy. Formulation and stability of freeze-dried proteins: Effects of moisture and oxygen on the stability of freeze-dried formulations of human growth hormone. *Dev. Biol. Stand.* 74:21–38 (1991).
15. K. Izutsu, S. Yoshioka, and Y. Takeda. The effects of additives on the stability of freeze-dried β-galactosidase stored at elevated temperature. *Int. J. Pharm.* 71:137–146 (1991).
16. L. Gatlin and P. P. DeLuca. A study of the phase transitions in frozen antibiotic solutions by differential scanning calorimetry. *J. Parent. Drug Assoc.* 34:398–408 (1980).
17. P. Labrude, B. Chaillot, F. Bonneaux, and C. Vigneron. Freeze-drying of haemoglobin in the presence of carbohydrates. *J. Pharm. Pharmacol.* 32:588–589 (1980).
18. K. Hellman, D. S. Miller, and K. A. Cammack. The effect of freeze-drying on the quaternary structure of L-asparaginase from *Erwinia carotovora*. *Biochim. Biophys. Acta* 749:133–142 (1983).
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275 (1951).
20. K. Izutsu, S. Yoshioka, and T. Terao. Stabilization of β-galactosidase by amphiphilic additives during freeze-drying. *Int. J. Pharm.* 90:187–194 (1993).
21. N. A. Williams and T. Dean. Vial breakage by frozen mannitol solutions: Correlation with thermal characteristics and effect of stereoisomerism, additives, and vial configuration. *J. Parent. Sci. Tech.* 45:94–100 (1991).
22. B. Debord, C. Lefebvre, A. M. Guyot-Hermann, J. Hubert, R. Bouche, and J. C. Guyot. Study of different crystalline forms of mannitol: Comparative behaviour under compression. *Drug Dev. Ind. Pharm.* 13:1533–1546 (1987).
23. K. Hayakawa. Freeze-thaw damage to *Saccharomyces cerevisiae* in saccharide solution. *Hakkokogaku* 63:9–15 (1985).
24. K. Seguro, T. Tamiya, T. Tsuchiya, and J. J. Matsumoto. Cryoprotective effect of sodium glutamate and lysine-HCl on freeze denaturation of lactate dehydrogenase. *Cryobiology* 27:70–79 (1990).
25. Y. Orii and M. Morita. Measurement of the pH of frozen buffer solutions by using pH indicators. *J. Biochem.* 81:163–168 (1977).